GM1 Specifically Interacts with α-Synuclein and Inhibits Fibrillation†

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ABSTRACT: The aggregation of α-synuclein is believed to be a key step in the etiology of Parkinson's disease. α-Synuclein is found in the cytosol and is associated with membranes in the presynaptic region of neurons and has recently been reported to be associated with lipid rafts and caveolae. We examined the interactions between several brain sphingolipids and α-synuclein and found that α-synuclein specifically binds to ganglioside GM1-containing small unilamellar vesicles (SUVs). This results in the induction of substantial α-helical structure and inhibition or elimination of α-synuclein fibril formation, depending on the amount of GM1 present. SUVs containing total brain gangliosides, gangliosides GM2 or GM3, or asialo-GM1 had weak inhibitory effects on α-synuclein fibrillation and induced some α-helical structure, while all other sphingolipids studied showed negligible interaction with α-synuclein. α-Synuclein binding to GM1-containing SUVs was accompanied by formation of oligomers of α-synuclein. The familial mutant A53T α-synuclein interacted with GM1-containing SUVs in an analogous manner to wild type, whereas the A30P mutant showed minimal interaction. This is the first detailed report showing a direct association between GM1 and α-synuclein, which is attributed to specific interaction between helical α-synuclein and both the sialic acid and carbohydrate moieties of GM1. The recruitment of α-synuclein by GM1 to caveolae and lipid raft regions in membranes could explain α-synuclein's localization to presynaptic membranes and raises the possibility that perturbation of GM1/raft association could induce changes in α-synuclein that contribute to the pathogenesis of PD.

α-Synuclein is an abundant, natively unfolded, and highly conserved presynaptic protein of unknown function, whose aggregation is implicated in the etiology of Parkinson's disease (PD). α-Synuclein is linked to the pathogenesis of Parkinson's disease through the discovery of familial missense mutants and duplication or triplication of the α-synuclein gene locus associated with early onset PD (*1*−*3*). The N-terminal region of α-synuclein, containing six 11-residue imperfect repeats, shares the apolipoprotein A2 lipid-binding helix motif (*4*, *5*). Several studies have demonstrated α-synuclein's association with lipids (*1*, *6*−*15*); however, the underlying molecular basis for this remains elusive.

α-Synuclein has been suggested to be associated with specialized neuronal membrane domains called caveolae or caveolae-like domains, since α-synuclein was found to regulate several signaling proteins found in these regions, including caveolin-1, extracellular signal-regulated kinase (ERK), PLD, and PKC (*16*−*18*). Recently, caveolae dysfunction was suggested to be involved in the pathogenesis of PD, due to α-synuclein's ability to upregulate caveolin-1 and downregulate ERK (*19*−*22*). However, the mechanism of α-synuclein accumulation in caveolae regions is unknown.

Caveolae are involved in numerous signaling functions and contain distinct morphological features, such as an enrichment of the caveolin family of proteins (*23*−*25*) and sphingolipids, mostly gangliosides and sphingomyelin. Lipid rafts are specialized plasma membrane microdomains that are enriched in cholesterol and sphingolipids (*26*, *27*), with similar lipid composition to caveolae (*28*−*30*). Interestingly, GM1 ganglioside has been proposed as a molecular marker for these regions and is specifically enriched in the caveolae (*31*, *32*). Gangliosides are sialic acid-containing glycosphingolipids consisting of a hydrophobic ceramide unit and a hydrophilic oligosaccharide. Sphingolipids, specifically glycosphingolipids, have been suggested to be the driving force for caveolae membrane domains (*33*−*35*) and can recruit proteins, e.g., caveolin-1 (*34*), to such regions. In this paper we propose a similar recruiting mechanism between GM1 and α-synuclein, which may account for α-synuclein's ability to interact with signal-regulating proteins located in caveolae-like regions and its involvement with synaptic membrane biogenesis and catecholamine regulation. Support for this hypothesis comes from a recent study showing that α-synuclein associates specifically with lipid rafts, suggesting an important role for rafts in the normal function of α-synuclein and raising the possibility that perturbation of raft association could induce changes in α-synuclein that contribute to the pathogenesis of PD (*36*).

In the present study, we examined the interactions between several brain sphingolipids and α-synuclein in vitro. We found that α-synuclein preferentially binds to small unilamellar vesicles (SUVs)[1] containing GM1 but not other

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[1] Abbreviations: ThT, thioflavin T; PL, phospholipid; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; NBD, 4-chloro-7-nitrobenzofurazan; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine

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sphingolipids, resulting in substantial α-helical structure and inhibition or elimination of fibril formation, depending on the amount of GM1 present.

MATERIALS AND METHODS

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and other phospholipids, total gangliosides, GM1 and related gangliosides, brain D-erythrosphingosine, brain sphingomyelin, brain sulfatide, brain ceramides, brain cerebrosides, brain total lipid extract, and NBD- (4-chloro-7-nitrobenzofurazan-) labeled DPPC were purchased from Avanti Polar Lipids. Laurdan was obtained from Molecular Probes, Inc. Thioflavin T (ThT) was purchased from Sigma, Inc., and was used without further purification.

Expression and Purification of α*-Synuclein.* Recombinant human wild-type α-synuclein was expressed in the *Escherichia coli* BL21(DE3) cell line transfected with pRK172 α-synuclein WT plasmid (kind gift of M. Goedert, MRC Cambridge) and purified by a procedure modified from ref *37*. Briefly, the pellet from 2 L of cells induced with 0.5 mM isopropyl *β*-D-thiogalactopyranoside was lysed by sonication at 0 °C in 50 mM NaCl, 20 mM Tris-HCl, 0.10% (v/v) Triton X-100, and 0.20 mM phenylmethanesulfonyl fluoride at pH 8.0. The lysis suspension was brought to 30% saturation with ammonium sulfate at 0 °C (pellet discarded) followed by 50% saturation with ammonium sulfate. The resultant pellet was dialyzed against 50 mM NaCl and 20 mM Tris-HCl, pH 7.5, loaded onto a 25 × 130 mm DEAE-Sepharose fast-flow column (Amersham Pharmacia Biotech) equilibrated in the same buffer, and eluted with a 50−450 mM NaCl gradient. Fractions containing α-synuclein were dialyzed exhaustively against water, clarified by centrifugation, and lyophilized for storage at −20 °C. The resultant α-synuclein protein was judged to be >95% pure following SDS−polyacrylamide electrophoresis, gel filtration, and MS analysis. A stock solution of purified α-synuclein was briefly treated with 1 mM NaOH, neutralized, and centrifuged for 30 min at 120000*g* before use to remove any aggregated material.

Preparation of Small Unilamellar Vesicles (SUVs) by Sonication. SUVs of experimental lipid/DPPC and DPPC alone were prepared by sonication as described previously (*38*). Briefly, SUVs of experimental lipid/DPPC (molar ratio 1:1, unless otherwise noted) and DPPC alone were prepared by sonication. Lipids, dissolved in chloroform stock solutions or a 60:40 ratio of chloroform/methanol, were mixed and dried under N_2 gas, forming a thin film on the wall of the vial. The thin film was hydrated in 100 mM NaCl and 20 mM Tris buffer, pH 7.4, with gentle mixing. Hydrated samples were sonicated at 4 °C with a probe sonicator at 30% energy. The sonicator program was set for 4 min at 10 s pauses after a 10 s sonication period to prevent overheating. Monomeric protein was added after sonication. Typical experiments used a mixture of SUVs with α-synuclein in a 10:1 mass ratio of lipid to protein.

Circular Dichroism (CD) Measurements. CD spectra were obtained from 195 to 250 nm, in a 0.01 cm cell, using an Aviv Model 60DS (Lakewood, NJ) spectrometer. Protein concentrations were kept at 0.8 mg/mL (55 μM), with the mass ratio of protein to lipid kept at 1:10. An average of five scans was taken for each sample, and background spectra of buffer and buffer with vesicles were subtracted to obtain the final spectra. Helical content was estimated using the program KD2.

Fibril Formation. Fibril formation was monitored while incubating 56 μM (0.8 mg/mL) protein, with and without vesicles, in the presence of 20 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl, and 20 μM thioflavin T, on a Fluoroskan Ascent, Thermo-Labsystems, 96-well plate reader. Five replicates of each set of conditions were run, and the results were averaged. ThT excitation was at 450 nm and emission at 485 nm. Fluorescence changes were plotted as a function of time, and lag times were calculated by curve fitting. Fibril formation was confirmed by EM.

Transmission Electron Microscopy. Samples were applied to carbon-coated pioloform copper grids, washed with water twice, negatively stained with 1% (w/v) uranyl acetate, and then visualized with a transmission electron microscope operating at 85 kV.

AFM Measurements. AFM imaging was performed with an Autoprobe CP Multiple AFM (Park Scientific) in non-contact mode as described (*39*). Aliquots of 2 μL of 200 mM NaCl were placed on a freshly cleaved mica substrate and incubated for 2 min. Aliquots of 3 μL of sample were placed on mica and incubated for 10 min. The substrate was rinsed with water to remove buffer, salt, and loosely bound protein and lipids and was then dried in air for 3 days. Heights ranging from 1.0 to 200 nm were estimated by section analysis, and lateral sizes were calibrated with various standard gold colloid particles.

Size Exclusion HPLC Measurements. SEC HPLC was as described (*40*). Briefly, sample volumes of 18 μL of supernatant from incubations were injected on a TSK-GEL G2000SWXL size exclusion column (7.8 mm i.d., 30 cm long) and eluted at a flow rate of 0.4−0.5 mL/min in 50 mM phosphate buffer, pH 7.0, and 100 mM sodium sulfate. HPLC was performed using a Waters 2695 separation module with a Waters 996 photodiode array (PDA) detector. Data were collected and analyzed by Millennium software. Absorbance of the eluate was monitored over the wavelength range of 220−500 nm with a bandwidth of 1.2 nm. Retention times were calibrated with protein molecular mass standards: ribonuclease A (13.6 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (bovine serum) (67 kDa), aldolase (158 kDa), and Blue Dextran 2000 (2000 kDa).

RESULTS

α*-Synuclein Shows Specificity for Binding to GM1*. α-Synuclein is known to bind to acidic phospholipid vesicles, adopting an α-helical secondary structure that can be observed by far-UV circular dichroism. In contrast, there is negligible interaction with vesicles of neutral phosphatidylcholin. Interestingly, the induction of helix and inhibition of fibrillation correlate with the relative concentrations of protein and lipid: at high protein to lipid ratios acceleration of fibrillation and no helical structure is observed, whereas at high lipid to protein ratios, helix formation and inhibition of fibrillation are seen (*38*). In order to measure whether α-synuclein bound to various brain sphingolipids, we performed circular dichroism (CD) experiments on α-synuclein in the presence of SUVs containing a 1:1 mixture of DPPC with the following sphingolipids: GM1, total brain

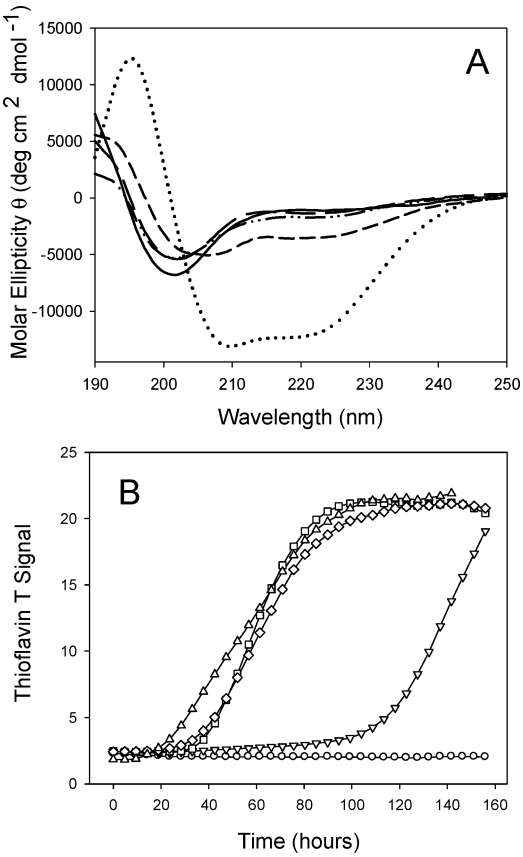

FIGURE 1: Effect of sphingolipid-containing vesicles on the secondary structure and fibrillation kinetics of α-synuclein. (A) Far-UV CD spectra of α-synuclein (solid line) in the presence of SUVs containing DPPC and cerebrosides (large dashed line), ceramides (dash-dotted line), total brain gangliosides (small dashed line), and GM1 (dotted line). (B) Kinetics of α-synuclein fibrillation monitored by ThT fluorescence: α-synuclein alone (triangles); α-synuclein in the presence of SUVs containing DPPC and cerebrosides (diamonds), ceramides (squares), total gangliosides (inverted triangles), and GM1 ganglioside (circles).

gangliosides, sphingomyelin, D-erythrosphingosine, ceramides, sulfatides, and cerebrosides at a mass ratio of 10:1 (total lipid to protein). This ratio was chosen since our previous studies had shown that such a ratio typically maximizes the helical structure of α-synuclein, suggesting saturation (*38*). The CD spectrum of α-synuclein with SUVs of DPPC alone exhibited a typical unfolded structure, with maximum negative ellipticity at 198 nm, confirming the absence of significant interaction with DPPC SUVs (Figure 1A). On the other hand, when GM1 was present in the SUVs, the CD spectra of α-synuclein displayed significant negative molar ellipticity at 206 and 222 nm and a large positive molar ellipticity at 195 nm, indicating formation of substantial α-helical secondary structure (∼50%) (Figure 1A). In order to assess the affinity of GM1 for α-synuclein, the CD spectra were measured as the concentration of GM1 in the SUVs was varied. The maximum ellipticity at 222 nm was observed with ∼50% GM1 in total brain lipid extract (see below), around −20000 deg cm^2 dmol^{-1}; with larger amounts of GM1 a decreased negative ellipticity was observed, i.e., less helicity. In addition, incubation of the samples at 37 °C led to increased helical structure with increasing time of incubation. Lipid rafts typically contain about 25−50% sphingolipids (*41*).

When α-synuclein was added to SUVs made from total brain gangliosides, the CD spectra exhibited a small amount of α-helical structure; all other sphingolipids had negligible effects on the secondary structure (Figure 1A). Total brain gangliosides consist mostly of various GT and GD gangliosides, with a small fraction of GM1 and negligible amounts of GM2 and GM3 (*42*). Thus, based on the induction of helical structure, α-synuclein exhibits very strong preferential binding to GM gangliosides, especially ganglioside GM1 (see below).

The effects of sphingolipid-containing SUVs on the fibrillation of α-synuclein were monitored with thioflavin T (ThT) fluorescence assays: ThT is a dye whose fluorescence emission increases greatly on binding to amyloid fibrils (*43*, *44*). These assays confirmed the correlation that some helical structure in α-synuclein inhibits fibrillation, while substantial helicity eliminates fibrillation completely (*38*). α-Synuclein binding to GM1/DPPC SUVs prevented fibril formation, whereas binding to total brain gangliosides/DPPC SUVs increased the kinetic lag time from 12.5 to 105 h, indicating a substantial inhibitory effect (Figure 1B). All other brain sphingolipids tested showed negligible changes in fibrillation kinetics compared to the control: data for the cerebrosides and ceramides are shown as examples of other sphingolipids in Figure 1.

In order to investigate the specificity toward GM1 in more detail,we examined the interaction of α-synuclein with SUVs containing closely related gangliosides, namely, GM2, GM3, and asialo-GM1. GM2 and GM3 have respectively one and two fewer sugars, and asialo-GM1 lacks the sialic acid moiety of GM1 (Figure 2). Gangliosides GM1, GM2, and GM3 all have one sialic acid group, leading to a net negative charge on the sphingolipid. Vesicles of DPPC containing 20% ganglioside were prepared and incubated with α-synuclein in a 10:1 mass ratio of lipid to protein. The far-UV CD spectra were collected (Figure 3A) and analyzed. Figure 3B shows the fraction of helical α-synuclein present: the amount of helix was maximal with GM1 and decreased in the order GM1 > GM2 > GM3 > asialo-GM1. Comparison of the amount of helix in the asialo-GM1 vesicles with those containing an equivalent amount of DPPE shows substantially more helix in the presence of the ganglioside, even though both are neutral. These results demonstrate that both the sialic acid group and the number of sugars present are critical in the interaction with α-synuclein. The not insignificant amount of helix induced in α-synuclein in vesicles containing ganglioside asialo-GM1 indicates that the sugar's head groups are important determinants of α-synuclein binding. This means that hydrogen bonding, as well as electrostatic interactions, is important in the transformation of natively disordered α-synuclein into its helical conformation on binding to lipids.

Association of α*-Synuclein with Sphingolipid/DPPC SUVs Observed by Size Exclusion HPLC.* In order to study the association between α-synuclein and sphingolipid SUVs, SEC HPLC analyses were performed on samples incubated at room temperature for 10−20 min. Absorbance readings were taken at 274 nm in order to locate protein, while detection of vesicles was made by incorporating a trace amount of the lipid fluorescence label NBD, with maximum absorbance values at approximately 340 and 480 nm. The SEC HPLC traces indicated complete binding of α-synuclein

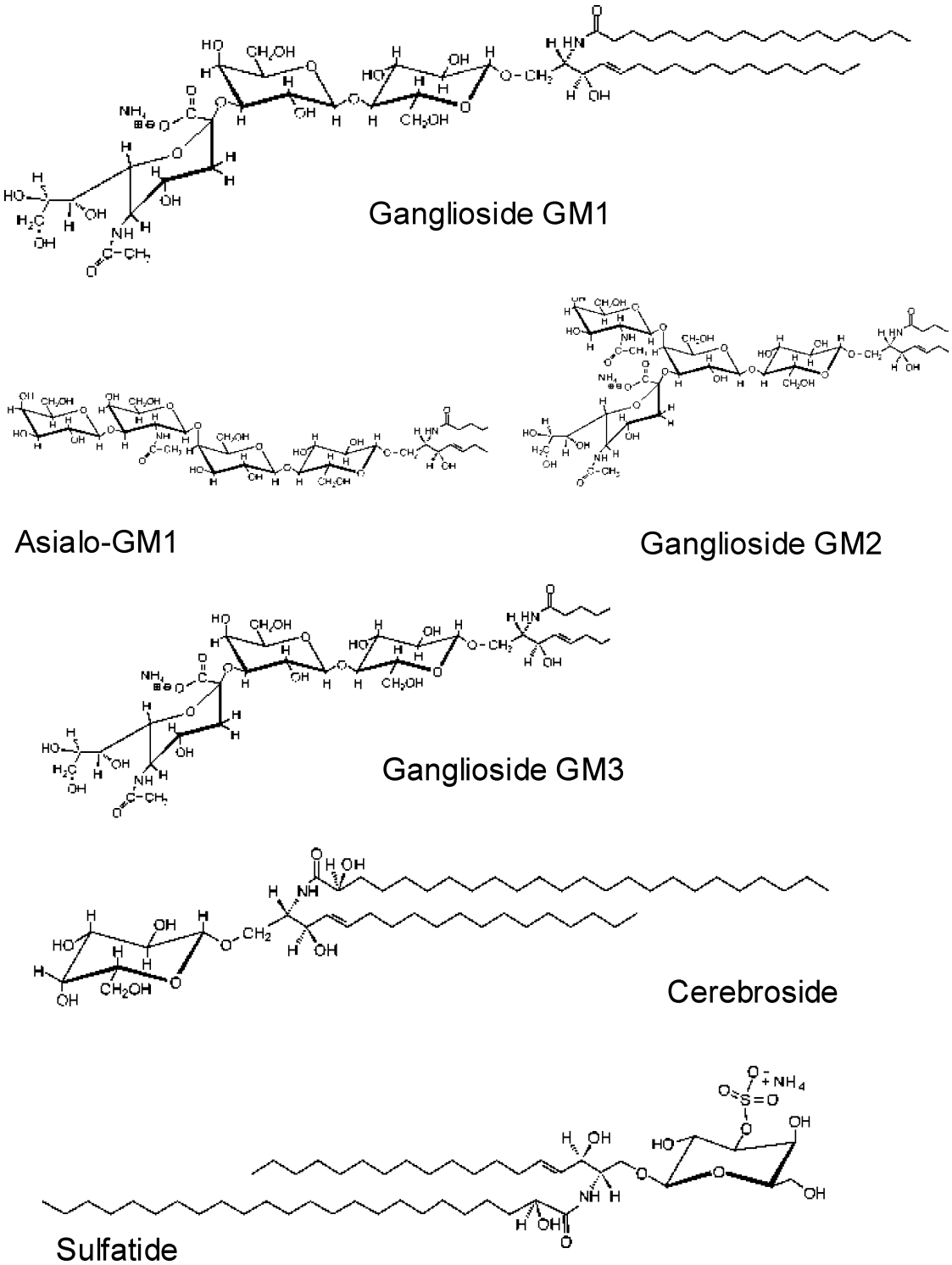

FIGURE 2: Structures of some of the sphingolipids investigated. The side chains have been truncated for gangliosides GM2, GM3, and asialo-GM1.

to GM1/DPPC SUVs, shown by a shift of the elution time from 23.75 min for α-synuclein alone to 18.5 min for α-synuclein bound to GM1 SUVs labeled with NBD (Figure 4). All the lipid was located in the elution peak at 18.5 min, as all of the NBD label was found in that peak (Figure 4, inset). Control experiments included running the vesicles alone and monitoring sphingolipid elution at 220 nm.

No association between α-synuclein and SUVs containing each of the other sphingolipids (excluding GM2, GM3) investigated was observed by SEC, with the only protein-containing fraction corresponding to the monomer elution peak, which showed the absence of lipid (based on the NBD chromophore). The early elution of α-synuclein in the presence of the GM1-containing SUVs could reflect elution of α-synuclein as an oligomer, an increase in α-synuclein size due to the bound lipid, or a combination of these. That the latter is the case is supported by subsequent data and the presence of the lipid probe in the eluting fraction.

GM1 Vesicles Induce the Formation of α-Synuclein Oligomers. Atomic force microscopy was used to analyze

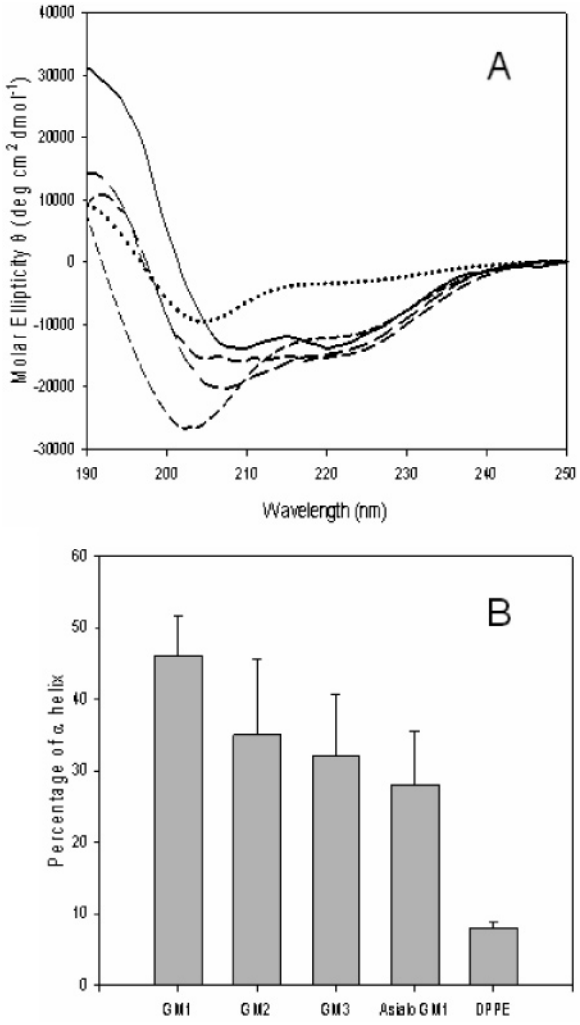

FIGURE 3: Effect of GM gangliosides on the secondary structure of α-synuclein. (A) Far-UV circular dichroism spectra of vesicles containing 20% ganglioside, 80% DPPC, and a 10:1 lipid to α-synuclein ratio: monomeric α-synuclein, dotted line; ganglioside GM1, solid line; asialo-GM1, short dashed line; ganglioside GM3, long dashes; ganglioside GM2, medium dashes. (B) Fraction of helical structure in α-synuclein in the presence of vesicles containing 20% of the indicated sphingolipids or dipalmitoylphosphatidylethanolamine (DPPE).

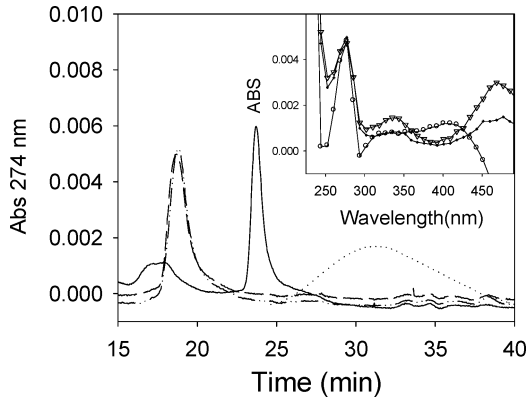

FIGURE 4: Binding of α-synuclein to sphingolipid/DPPC vesicles monitored by size exclusion HPLC: α-synuclein alone (solid line), bound to 2.5% NBD-containing vesicles (dashed lines), 5% NBD-containing vesicles (dash-dotted line), and 2.5% NBD-containing vesicles without α-synuclein (dotted line). Inset: Absorbance spectra corresponding to peaks in the SEC HPLC trace: 5% NBD (inverted triangles); 2% NBD (filled dots); α-synuclein alone (circles).

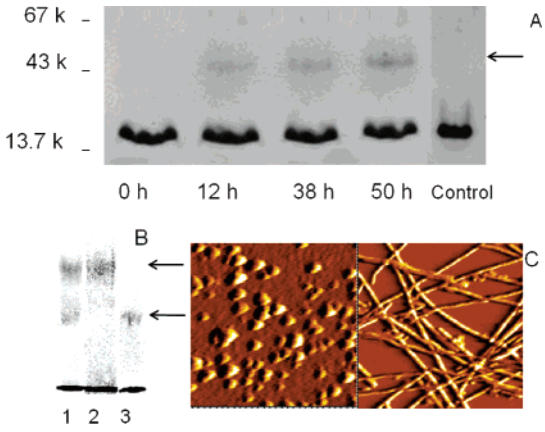

FIGURE 5: Oligomerization of α-synuclein after incubation with GM1-containing SUVs. (A) SDS−PAGE of α-synuclein incubated in the presence of GM1-containing SUVs; the arrow points to the oligomers. (B) Native PAGE of WT (lane 1), A53T (lane 2), and A30P (lane 3) showing α-synuclein oligomers (arrows). Samples were taken after 140 h incubation in the presence of GM1-containing SUVs. (C) AFM image of oligomers of α-synuclein after incubation: left, in the presence of GM1 vesicles; right, in the absence of vesicles. See text for details.

the nature of the bound α-synuclein after incubation with GM1/DPPC vesicles. As seen in Figure 5C (left), small spherical particles of α-synuclein of slightly varying size were observed. From analysis of particle heights the size distribution was as follows: 21% at 0.75 nm, 39% at 1.1 nm, 25% at 1.6 nm, and 14% at 2.6 nm height. In solution, monomeric α-synuclein has a hydrodynamic diameter of ~0.7 nm (*45*, *46*), suggesting that the smallest observed species here are monomers. The larger species could reflect either oligomeric forms of α-synuclein or α-synuclein with associated lipid. Since the SUVs are ~25 nm (confirmed by EM), the species seen in the AFM images are not the vesicles. Figure 5C (right) also shows an AFM image of fibrils formed in a control experiment in which α-synuclein was incubated in the absence of GM1 (similar images are seen when α-synuclein is incubated in the presence of vesicles containing other non-ganglioside lipids).

The larger species were not present before incubation of the vesicles with α-synuclein and became more stable as the incubation progressed, as indicated by the appearance of a band at approximately 43 kDa seen by SDS−PAGE gel (Figure 5A). Two oligomer bands were observed on native gels after incubation with GM1-containing SUVs with α-synuclein, indicating the formation of two distinct stable oligomers (see Figure 5B, lane 1). None of the other sphingolipids (excluding GM2, GM3), including total gangliosides, induced detectable stable oligomeric structures on native gels or SDS−PAGE gels (data not shown). The size of the oligomers observed on SDS−PAGE suggests that they are stable dimers.

Total Brain Lipid Extract Induces α-Synuclein Fibril Formation, but GM1 Induces α-Helical Structure and Eliminates Fibrillation. Previous investigations have found lipid-binding specificity between acidic phospholipids such as phosphatidylserine (PS) and α-synuclein (*47*). This led us to study the interactions between total brain lipid extract, which consists of approximately 16% PE and 10% PS, and α-synuclein. We found that when SUVs were composed of 100% total brain lipid extract, at a concentration of 275 μM,

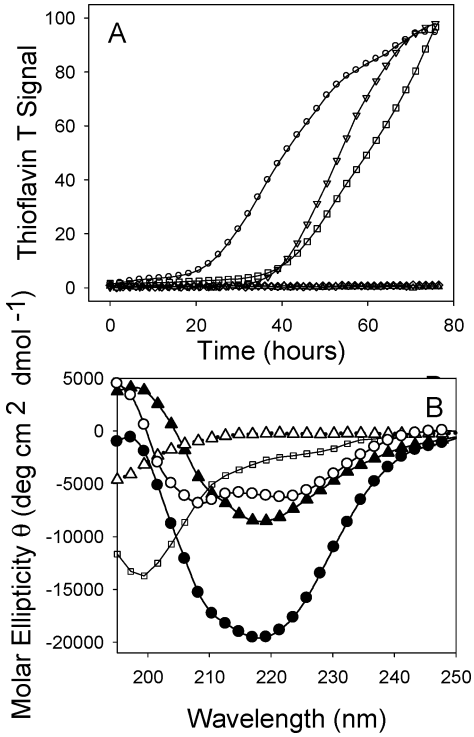

FIGURE 6: Effect of total brain lipid extract vesicles, alone and with GM1, on the fibrillation kinetics and secondary structure of α-synuclein. (A) Kinetics of α-synuclein fibrillation monitored by ThT fluorescence: α-synuclein alone (inverted triangles); α-synuclein in the presence of SUVs containing 100% total lipid brain extract alone (circles), 20% GM1/80% brain extract (squares), 50% GM1/50% brain extract (diamonds), and 80% GM1/20% brain extract (triangles). (B) Far-UV CD spectra of aqueous monomeric α-synuclein (squares), α-synuclein and SUVs of total lipid brain extract with 20% GM1, before (open triangles) and after incubation (filled triangles), and with 50% GM1, before (open circles) and after incubation (filled circles). In the latter case prior to incubation the α-synuclein is predominantly α-helical; after incubation it becomes predominantly β-sheet due to the presence of the oligomers.

they accelerated α-synuclein (55 μM) fibril formation; the lag time decreased from 37.5 to 18.75 h (Figure 6A), and fibrils were seen in abundance by transmission electron microscopy (TEM) (Figure 7B). Far-UV CD showed an increase in molar ellipticity at 198 nm, compared to the control; however, no significant secondary structure was observed (Figure 6B), i.e., no helix induced. In order to investigate the effect of GM1 on this process, GM1 was added to SUVs of total brain lipid extract in incremental amounts from 20% of total lipid to 80%, with the total lipid concentration remaining 275 μM. Upon addition of 20% GM1 the lag time for fibrillation increased from 18.8 h for total brain extract alone to 38.8 h (Figure 6A), while the far-UV CD showed no significant induction of secondary structure (Figure 6B), and fibrils were visible by TEM (panels B and C of Figure 7, respectively). At a lipid composition of 50% GM1 fibrillation was completely eliminated, as observed by no increase in the ThT fluorescence signal and a lack of fibrils by TEM (Figure 7D). These samples showed the presence of α-helical structure before incubation (Figure 6B), which is in accord with previous results showing that when α-synuclein is in an α-helical conformation, fibrillation is inhibited or eliminated (*38*). Interestingly, incubation of α-synuclein in the presence of

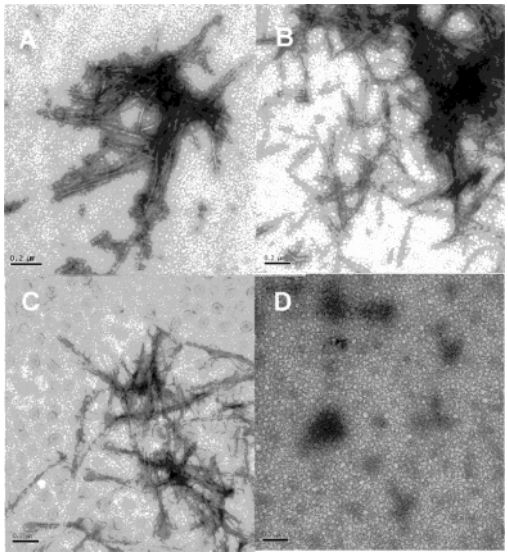

FIGURE 7: Electron micrographs showing the relative amounts of fibrils corresponding to vesicle composition. All samples contained WT α-synuclein at 0.8 mg/mL. Panels: (A) without SUVs; (B) 100% total lipid brain extract SUVs; (C) 20% GM1/80% total lipid brain extract SUVs; (D) 80% GM1/20% total lipid brain extract SUVs. The scale bars represent 200 nm.

the GM1-containing vesicles leads to the appearance of β-structure in the CD spectra (Figure 6B), indicating that the oligomers contain significant β-sheet structure.

Interactions of the Familial PD Mutants A53T and A30P with GM1/DPPC SUVs. SUVs of sphingolipid/DPPC were prepared, and wild-type, A30P, and A53T α-synuclein were added at a 10:1 lipid to protein ratio. Fibrillation kinetics were monitored by ThT; the results were as expected for samples without lipid (*48*); lag times increased in the order A53T < WT < A30P α-synuclein (data not shown). When GM1/DPPC SUVs were added, however, fibrillation was eliminated for WT and A53T, but not so significantly for A30P (Figure 8A), and induction of α-helical secondary structure was observed only for samples of WT and A53T (Figure 8B). The low value for A30P fibril formation in the absence of GM1 in Figure 8A reflects, in part, the slow kinetics of fibrillation. A native gel was run after incubation, which indicated the existence of two major protein bands for WT α-synuclein and only one band for each of the two mutants (Figure 5B). Interestingly, it appears that the A30P mutant forms the smaller species found in the WT lane, while the A53T mutant forms the larger species.

DISCUSSION

Previous investigations have demonstrated α-synuclein's ability to bind to acidic phospholipid-containing vesicles, with an associated induction of α-helical secondary structure (*7*, *38*, *47*, *49*−*53*) and inhibition of fibril formation (*38*). It has also been shown that α-synuclein interacts with certain lipids found in the brain more than others, specifically the phospholipids PE, PA, PI, and PS, suggesting a similar affinity in vivo and in vitro (*7*, *38*, *47*). There is extensive literature on the potential involvement of GM1 gangliosides in Parkinson's disease and the beneficial effects of GM1 supplementation in animal models of the disease (*54*−*60*). Among other effects, ganglioside GM1 is able to counteract the effects of damage to the dopamine system (*61*) and to

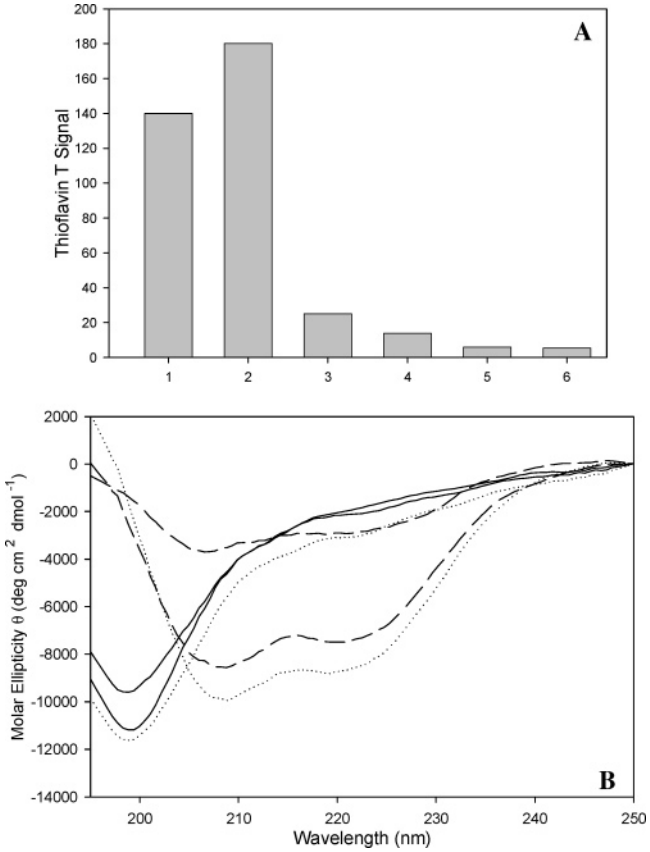

FIGURE 8: Effect of GM1/DPPC vesicles on the fibrillation kinetics and secondary structure of WT, A30P, and A53T α-synuclein. (A) Comparison of thioflavin T signals of protein with and without GM1/DPPC vesicles after 140 h incubation. Bars 1, 2, and 3 are WT, A53T, and A30P without GM1/DPPC SUVs; bars 4, 5, and 6 are WT, A53T, and A30P with GM1/DPPC SUVs. (B) Far-UV CD spectra of WT α-synuclein (dotted lines), A53T α-synuclein (dashed lines), and A30P (solid lines) in the absence or presence (spectra with lowest ellipticities at 220 nm) of GM1/DPPC SUVs.

rescue nigral neurons and increase dopamine synthesis in residual nigrostriatal dopaminergic neurons that have been treated with MPTP (*62*), and anti-GM1 antibodies have been found at elevated levels in patients with Parkinson's disease (*59*). There is also evidence for a connection between some glucocerebrosidase disorders, such as Gaucher's disease, and GM1 ganglioside and Parkinson's disease (*59*, *63*−*68*). Accumulation of GM2 ganglioside is associated with accumulation of α-synuclein in neurons of patients with Sandhoff disease, a lysosomal storage disease (*69*). Binding of α-synuclein to human brain-derived gangliosides has also been reported (*70*), and as noted, α-synuclein can localize to lipid rafts and caveolae, which are rich in GM1 ganglioside (*19*, *32*, *36*). Recently, it has been shown that, in vesicles composed of PS, cholesterol, and sphingolipids in the 1:1:1 ratio typically found in lipid rafts, the ratio of unsaturated (or monounsaturated) and polyunsaturated fatty acids in the phospholipid was critical in determining the binding affinity of α-synuclein (*71*).

In the current study we demonstrate α-synuclein's specificity toward sphingolipid GM1, a member of a class of lipids abundantly expressed in the brain and concentrated in caveolae and lipid raft regions (*72*−*74*). Among the six classes of brain sphingolipids we studied (total brain gangliosides; gangliosides GM1, GM2, and GM3, asialo-GM1; sphingomyelin; cerebrosides; ceramides; sulfatides; and D-erythrosphingosine) only the gangliosides exhibited any significant effect on α-synuclein structure or fibrillation kinetics, and the effects with ganglioside GM1 were far more pronounced. This indicates that the presence of an oligosaccharide is required for α-synuclein binding. The results also indicate that α-synuclein shows very strong specificity toward GM1 even when compared to the other GM gangliosides. Samples of α-synuclein-containing GM1/DPPC SUVs showed a substantial increase in α-helical structure, the absence of fibril formation, and strong affinity for the vesicles as observed by SEC HPLC, whereas corresponding SUVs of total brain gangliosides/DPPC induced helical structure to a lesser extent and only weakly inhibited fibril formation as seen by an increase in the lag time in the ThT assays. Furthermore, size exclusion HPLC data of total gangliosides showed negligible stable binding of protein to the SUVs. This finding is notable due to the close structural similarity between total brain gangliosides and GM1; the carbohydrate moieties of gangliosides are divergent by only a few sugar groups. All other sphingolipids, which have a common ceramide moiety with gangliosides, were found to have negligible affinity for α-synuclein by SEC HPLC, circular dichroism, and effects on fibrillation kinetics. These results indicate that among the brain sphingolipids α-synuclein shows uniquely strong binding specificity for GM1 (and significantly lesser affinity for gangliosides GM2 and GM3) and that this may account for its localization to presynaptic membranes, since gangliosides are concentrated in synaptic terminals.

The effects of α-synuclein binding to GM1/DPPC SUVs compared to binding to corresponding total brain ganglioside/DPPC SUVs, and especially other brain sphingolipids, are striking, in that our data show that only GM gangliosides, and specifically GM1, inhibit fibrillation. This parallels the corresponding induction of helical structure. As noted, lipid rafts typically contain about 25−50% sphingolipids (*41*). Our results comparing GM1 with asialo-GM1 and GM2 and GM3 demonstrate that both the sialic acid group and the number of sugars present are critical in the interaction with α-synuclein. Thus, the fact that the sugar headgroups are important determinants of α-synuclein binding means that hydrogen bonding, as well as electrostatic interactions, is important in the binding of α-synuclein to membranes.

We propose that this specificity of α-synuclein for GM1 arises from the unique spatial orientation of the carboxylate groups of the sialic acid components of GM1, especially when it is aggregated as in lipid rafts, which permits interaction with the positively charged Lys side chains of α-synuclein in its helical conformation. In addition, specific H-bonding between sugar hydroxyls of GM1 and side chains of α-synuclein are clearly also very important. The differences in location and orientation of the sialic acid and carbohydrate hydroxyls in the different gangliosides must account for the difference in affinity between ganglioside GM1 compared to GM2 and GM3 and demonstrate that both sialic acid position and oligosaccharide structure make significant contributions to the specificity of α-synuclein binding. The fact that asialo-GM1 still showed some affinity for α-synuclein indicates that it is not only the negative charge on the head group that is important for α-synuclein binding but also the sugars. Since cerebrosides have a single

sugar headgroup, it is clear that multiple sugars are required for α-synuclein affinity. The fact that vesicles containing sulfatides, with their negatively charged headgroup, failed to bind α-synuclein indicates that even a single negatively charged sugar is insufficient for α-synuclein affinity. In light of the recent report by Kubo et al. (*75*) demonstrating maximal affinity of raft-like vesicle compositions containing PS with mixed unsaturated (or monounsaturated) and polyunsaturated fatty acids, we expect that in the presence of vesicles of PS with both mono- and polyunsaturated fatty acids chains there would be even stronger affinity of α-synuclein for GM1-containing vesicles and presumably also with lipid rafts.

The markedly decreased interaction of the A30P familial mutant observed with GM1 [and phospholipids in general (*76*)] may be accounted for by the Pro-induced disruption of the helical structure, which is not present in the A53T mutant, which has a comparable interaction with GM1 to wild type. This suggests that the region of α-synuclein in the vicinity of residue 30 plays an important role in membrane association.

The AFM, SEC HPLC, and PAGE data are all consistent with the formation of stable α-synuclein oligomeric species formed during incubation at 37 °C with GM1/DPPC SUVs. The formation of oligomers was unique to samples containing GM1 SUVs and not other non-GM sphingolipids. Since the CD spectra indicate high helical content, this observation suggests that the oligomers involve the association of helical α-synuclein on the membranes.

On the basis of our results it is reasonable to propose an interaction between α-synuclein and ganglioside GM1 that would lead to the accumulation of α-synuclein in cell membrane regions rich in gangliosides such as the caveolae- and raft-like membrane domains in synaptic terminals. The localization of α-synuclein to lipid rafts has recently been reported in vivo (*36*) and undoubtedly is an important component of the function of α-synuclein. This hypothesis may have neurological significance considering that gangliosides enriched in GM1 are highly expressed and abundant in neurons, where they can comprise more than 10% of total lipid in synaptic regions and perhaps as much as 40−50% in specialized domains such as caveolae and lipid rafts due to their unique ability to self-associate (*77*−*80*). Previous investigations have linked GM1 and GM1-enriched membrane domains to PD, while others have linked α-synuclein to PD; however, this is the first detailed report showing a direct association between GM1 and α-synuclein. Interestingly, the Aβ polypeptide associated with Alzheimer's disease has been shown to bind to GM1-containing liposomes; however, in contrast to α-synuclein, this leads to the induction of β-structure and enhanced fibrillation (*81*−*85*). It is likely that the interaction with GM1 is related to the putative role of α-synuclein in trafficking of vesicles. In addition, there is some evidence that α-synuclein may not always be intracellular.

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